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BIOLOGICAL BULLETIN

THE BEHAVIOR OF THE NUCLEUS AND CHROMOSOMES DURING SPERMATOGENESIS IN THE ROBBER FLY *LASIOPOGON BIVITTATUS*.[1]

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INTRODUCTION.

During the growth period of the primary spermatocytes in *Lasiopogon bivattatus* Lw.,[2] nuclear changes occur which transform the spherical nucleus into a series of ramifying chambers enclosing the chromosome threads. Conditions in these nuclei suggest that the stainable threads are axes or cores of considerably larger bodies, which are mainly hyaline. The present account is concerned primarily with these features, although it includes a description of spermatogenesis up to the second spermatocyte division.

The observations are based on two specimens, collected near Sheridan, Wyoming. Both were fixed in strong Flemming and stained with iron hæmatoxylin. One (2055) was counterstained with light green. The technique employed was the same as that regularly used in preparing dipteran material, and the fixation is good in both specimens. So far as we know the peculiar nuclear behavior is characteristic of the species and does not represent an accidental abnormality.[3]

[1] A preliminary account of this was given before the Society for Experimental Biology and Medicine and published in the Society's Proceedings (see Metz and Nonidez, '22).

[2] We are indebted to Mr. Jas. R. Hine for the species determination.

[3] This is supported by the fact that conditions somewhat similar have been found in two other species of flies (discussed below). In these our material is sufficiently extensive to prove that the conditions are normal.

The testes of *Lasiopogon* resemble those of *Asilus* and many other members of the Asilidæ, in being a pair of long, coiled tubes, with the spermatogonia near the apex, and successive stages in spermatocyte development progressing in serial order toward the base. This serial orientation of stages makes it relatively easy to follow the succession of events during spermatogenesis.

Spermatogonia.

At the extreme tip of the testis lies a small mass of disorganized, nutritive material containing only a few cells. Adjacent to this lie the spermatogonia, many of which are connected with the nutritive mass by long protoplasmic processes. This condition differs from that in many other asilids. In the latter the nutritive mass lies in the center of the tube, a short distance from the end, and is surrounded on all sides by spermatogonia.

The spermatogonia present no unusual features except in late prophase. There the nuclei appear to undergo a change similar to that observed in the primary spermatocytes. The nuclear membrane breaks down or becomes irregular in outline and the chromosomes appear to lie free in the cytoplasm, each surrounded by a hyaline zone of about the thickness of the chromosome. The other stages in spermatogonial development appear to be essentially the same as those in *Asilus sericeus* (Metz and Nonidez, '21).

The spermatogonial chromosome group probably consists of six pairs, or possibly of five pairs of autosomes and an unpaired X-chromosome. Fig. 1 only shows five pairs, but the nucleus may not be entire. One or two other figures (not suitable for drawing) seem to include six pairs, and spermatocyte metaphases almost certainly include six chromosomes (*e.g.*, Fig. 12).

The Growth Period.

Owing to the scarcity of final spermatogonial division figures and to the fact that the final spermatogonia are not readily identified by size differences, as they are in some asilids, it is difficult to detect the boundary between spermatogonia and spermatocytes. However, the earliest growth stages seem to repeat the spermatogonial prophase changes, as in the case of *Asilus sericeus* (*l. c.*, p. 171); and so far as we can determine they show essen-

tially the same features as does *Asilus*. Hence they will be passed over rapidly.

The pairs of chromosomes appear soon after the final spermatogonial telophase as granular aggregates (Fig. 2). They are less dense and clear cut than in *Asilus*, and more nearly resemble those in *Dasyllis* (Metz, '22). These aggregates soon loosen up, overlap, and spin out into long, granulated threads, intermingled in a net-like structure. The nucleolus, a dense, spherical body, is prominent from the earliest observed stages (see special account of the nucleolus below).

Soon after the growth period has begun, the chromatin threads become drawn out so finely that they cannot be traced far, individually. They are heavily granulated, with prominent chromomeres. From their history and from their subsequent behavior, as well as from analogy with other forms, they are assumed to be bivalent, although no duality is visible in their structure. There is no conspicuous polarization of the threads at this time, nor is there any evidence of a synizesis or dense contraction stage. The nucleus is clear cut in outline and is of the usual more or less spherical shape, as is also the nucleolus.

At this point changes set in which accompany one another as growth progresses. The nucleolus elongates and eventually becomes double; the chromatin threads undergo a gradual condensation; and the smooth contour of the nucleus becomes broken by a series of invaginations and evaginations, at first more or less irregular, but soon conforming to the outline of the thread-like chromosomes. It is difficult to determine what physical changes occur in the cell; but the nucleus acts as if its tension were released, so that the nuclear membrane offered no resistance to movements of the cytoplasm or of the chromosomes. The former appears to flow in around the chromatin threads, while the latter become extended out into the cytoplasm. Soon the nucleus is converted into a series of long lobes or pockets ramifying throughout the cell. Each pocket, as a rule, encloses only one (bivalent) chromatin thread. These changes are represented by the cross sections shown in Figs. 2 to 4. The surface of the nucleus is tremendously increased by this process, but the volume is so difficult to estimate that we are unable to determine whether it is affected or not.

Little or no regularity of plan is to be seen in the configuration of the nucleus. Its shape is varied and often bizarre. Apparently the lobes ramify at random, following the contour of the long, more or less twisted threads. The nucleolus usually lies in a lobe or pocket by itself, or in a chamber at the junction of two or more lobes.

The lobes themselves are of various shapes, flat, pocket-like sheath-like, cylindrical, or of intermediate sorts. It is to be noted, however, that all of the nuclei undergo the same series of changes and show essentially the same features. There is no irregularity in this respect.

The maximum of distention and distortion of the nucleus is reached soon after the process begins, and while the chromatin threads are long and slender. At this time the lobes are so attenuated and slender that the nucleus is almost lost to view. For the most part it can only be seen as narrow, winding channels, ramifying through the cytoplasm. These channels often extend almost or quite to the periphery of the cell. Since each channel usually contains a single (bivalent) thread, the threads thus become almost completely isolated from one another.

Subsequently the threads undergo a gradual condensation, and the lobes a coincident contraction, as growth progresses (Figs. 5–9).

When the detailed structure of the lobes is examined, at any stage, it is seen that almost invariably the deeply staining chromatin thread (chromosome), extends through the center of the hyaline lobe like a core, if the lobe is cylindrical, or lies midway between the sides, if the lobe is flat. It is separated from the membrane by a transparent layer of approximately uniform thickness. The structure might be likened to an insulated wire, in which the chromosome represents the wire and the hyaline region the insulation.

As the lobe is followed beyond the chromosome it is seen to become narrow or closed. The maximum thickness is almost always in the region occupied by the chromosome. This suggests that the chromosome is surrounded by a cortex of dense or gelatinous material which holds off the nuclear membrane.

No essential change in these conditions is to be observed as the chromosomes condense and the lobes contract. The two

processes apparently go together. As the chromosome thickens, the surrounding layer thickens, increasing the diameter of the lobe in that region. This process gradually progresses up to the prophase, when, as the chromosomes approach their maximum condensation the lobes become decreased in size. In the late growth period there is a suggestion of a secondary lengthening out of the chromosomes and the lobes into a more ramifying condition such as exists at an earlier stage, but the change is not extreme, and may not be constant. It suggests, however, the stage in *Asilus sericeus* (*l. c.*) in which the threads spread apart after undergoing contraction. This also occurs relatively late in the growth period.

In *Lasiopogon* there is no "contraction stage," such as occurs in *Asilus;* but throughout most of the growth period there are some indications of a polarization of the threads toward the nucleolus; and often faint connections are to be seen between the two (Figs. 7 and 8).

During the prophase, and up to a late stage in metaphase, the hyaline layer around the chromosomes remains conspicuous, as shown in Figs. 10–13. The spindle fiber may often be traced to a projection of this layer corresponding to that of the chromosome, and thence through the layer to the chromosome.

Not enough anaphase figures are present in our material to permit us to follow the chromosomes through this stage.

During the late growth period and the first spermatocyte division the mitochondria become very prominent, and by darkening the cytoplasm, aid in bringing the outline of the chromosomal sheath into relief. In metaphase large mitochondrial rods are frequently present which are almost as dense as the chromosomes. These are seen longitudinally in Figs. 10 and 13, and in transverse section in Figs. 11 and 12. It is to be noted that they are not surrounded by a hyaline cortex comparable to that around the chromosomes.

The Nucleolus.

The nucleolar behavior during the growth period is somewhat different in our two specimens. In one (2056) there is a single nucleolus in the early growth stages,while in the other (2055) there are two. In the following description the two specimens are considered separately.

In number 2056 the nucleolus is prominent from the beginning of the growth period. It grows gradually and serves as an additional criterion for seriating the growth stages. When the nucleus begins to become irregular in outline, the nucleolus elongates and becomes bilobed, then double. One portion is large, dense and irregularly shaped; the other is smaller, less dense and nearly spherical—resembling chromatin in texture. The two parts are often connected by a dark, thin thread. In the late growth stages the large, irregular portion frequently lies near the periphery of the cell, suggesting that it may be cast off. We have not been able to determine its fate, on account of the scarcity of prophase figures, but it seems to disappear before the chromosomes go on the spindle. The smaller portion appears on the spindle in its characteristic spherical form, but reduced in size, and presumably represents the sex chromosomes.

In specimen 2055 there are normally two, equal sized, spherical nucleoli in the early growth stages. Just before the nucleus begins to become lobulated the two nucleoli come close together and apparently unite, although it is possible that one may degenerate after they become approximated. Following this the nucleolus becomes bilobed as in specimen 2056, and subsequently its history is the same as that of the latter.

In both specimens the nucleolar structures are surrounded by a hyaline cortical region similar to that of the autosomes, although it may be noted that the cortex of the large, irregular portion is usually thinner than that of the small, spherical part which probably represents the sex chromosomes.

Discussion.

The constancy of the hyaline zone around the chromatic threads in the nuclear lobes we interpret as indicating the presence of a relatively solid (gelatinous) cortical layer enveloping the respective chromosomes. The presence of the layer is revealed, of course, by the structure of the lobes. If the nuclei were spherical it would not be detected. This raises the question as to whether the condition is restricted to the present species, or is of more general occurrence but usually invisible.

We have noted above that evidence of the hyaline zone is to be seen in spermatogonia as well as in spermatocytes of *Lasiopogon*.

In the other Diptera known to us,[1] conditions are not usually such as to reveal the presence of a chromosomal envelope if one exists. But indications of one are to be seen here and there, especially in late prophases or metaphases. And in one genus, owing to the unusual nuclear behavior—somewhat like that in *Lasiopogon*—the evidence is strong. This genus is *Ptecticus*, a member of the Stratiomyidæ. It will be considered more fully in another paper, but we may note here that in *P. trivittatus* and *P. sackenii*, the two species we have studied, there is a distinct hyaline zone around the chromosomes of the prophase and metaphase of the spermatocyte divisions, and there are indications of such a structure extending back into the growth period. These features are shown in part by Figs. 14 and 15, from primary spermatocytes of *P. trivittatus*.

This condition, in a family distinct from that to which *Lasiopogon* belongs, suggests that we are dealing with something which is widespread—at least among the Diptera.

In other organisms the nearest approach to conditions like those described above, so far as we are aware, is found in the Orthoptera. Professor McClung informs us that in this group prophase and metaphase chromosomes (of primary spermatocytes) are frequently bordered by clear spaces, differentiated from the surrounding cytoplasm, and that the condition is sufficiently characteristic to suggest the constant presence of a hyaline zone around the chromosomes in these stages. As examples may be cited McClung's published photographs of the first spermatocyte chromosomes of *Mermeria* (McClung, '14, Figs. 129–132) and of *Hesperotettix* (McClung '17, plate 8, Fig. *c*). In all of these the hyaline zone is visible around the chromosomes.[2] We have observed this condition in first spermatocyte anaphases of *Rhomaleum* and find that the hyaline layer agrees in appearance with that in *Lasiopogon*, although we have not as yet tried to trace its history.

The presence of this condition in the Orthoptera and Diptera has prompted us to make a brief comparison with other structures of a somewhat similar nature.

[1] No close relatives of *Lasiopogon* have been examined as yet.

[2] It is probable that other cases of this sort have escaped our notice, although we have examined numerous published photographs of chromosomes with this point in mind.

These include, for the most part, the "chromosomal vesicles" of Conklin ('01, '02, etc.), Wenrich (16), Richards (17) and others. A resumé of the observations on these structures up to 1917 has been given by Richards (*l. c.*), who cites examples in various organisms from molluscs to vertebrates (fish eggs). There is considerable difference in the structure of the vesicles in different forms, but at certain stages in several, if not all, of them it consists of a hyaline outer layer surrounding a more or less definite chromatic axis, suggestive of the lobes in *Lasiopogon*. This is clearly the case in spermatogonial telophases of the Orthoptera and in *Fundulus* eggs (cf. Wenrich, Richards). The stages in which such a condition exists (prophases in the case of *Fundulus*) may be preceded by others in which the chromatic material is scattered through the vesicle, or even largely applied to the surface (*Crepidula*, Conklin '02). In *Fundulus* eggs and in the Orthoptera the anaphase chromosome swells into a vesicle in which the chromatin appears as a granulated network. This persists through the resting stage and then the network condenses into a chromatic thread or rod, extending more or less axially through the vesicle.

If we assume that the hyaline portion of the vesicle is more dense or gelatinous than the surrounding cytoplasm, as is suggested by the appearance and behavior of the vesicles, then the structure bears a close resemblance to that in *Lasiopogon*. On this basis we might conceive of the structures in *Lasiopogon* as arising in a similar fashion—*i.e.*, by the swelling of telophase chromosomes into vesicles, with a diffusion or dispersal of the chromatin (as seen in the early growth stages) and then its recondensation within the vesicle. The term "vesicle" is perhaps inexact in the case of *Lasiopogon* for no limiting membrane, except the nuclear membrane, is visible around the hyaline layer. But the periphery of the latter would, on this view, be considered as analogous to the periphery of the vesicle, whether enveloped in a membrane or not.

The only other description of a structure similar to these, so far as we know, is that of Lee's "chromosomal sheath."[1] In the case of the Orthoptera cited by Lee his sheath evidently corre-

[1] We are indebted to Professor L. W. Sharp for calling our attention to Lee's paper and to de Litardière's criticism of it mentioned below.

sponds to the hyaline portion of the vesicle considered above, and as such is similar to that in *Lasiopogon*. But his conception of a sheath as characteristic of animal chromosomes and possibly also of plant chromosomes, seems to us to be open to serious question, at least in the form stated. It has been considered at length in a recent paper by de Litardière ('21), who interprets the "sheath" as an artifact—probably a fixation product. The latter author maintains that the same sort of zone is to be seen around the mitochondria, cytoplasmic inclusions, etc., and hence has no special connection with chromosomes. We may omit further consideration of these structures, therefore, until the evidence becomes clearer.

Possibly de Litardière's criticism may apply to the cases we have cited here of "indications" of a hyaline zone around chromosomes in the Diptera, including *spermatogonial* chromosomes of *Lasiopogon;* and it may apply to the zone seen in McClung's figures referred to above. Further evidence is required to settle this point. But we may note that in these cases the zone seems to be found only around the chromosomes, not around other structures in the cell.

It may be added that in the case of the *spermatocytes* of *Lasiopogon* described here the structures are clearly not artifacts, and are confined to the chromosomes (including the nucleolus which, from analogy with other Diptera, is assumed to be chromosomal in nature). Numerous large mitochondrial rods are present, but are not surrounded by sheaths comparable to those enveloping the chromosomes.

If we omit the questionable cases, such as those just mentioned as possibly due to artifacts, it is clear that few forms exhibit structures comparable to those in *Lasiopogon*, although the possibility still remains that the structures may be *present* in others even when not visible.

In the foregoing discussion little emphasis has been laid on the fact that some of the cases considered involve bivalent chromosomes (maturation stages) and others univalent chromosomes. It is not yet clear that any distinction should be made, so far as the present subject is concerned. But it may be noted in closing that any view which considers the chromosome as enclosed in a sheath or envelope must take account of the approximation of

homologous chromosomes during synapsis, and, in the case of the Diptera, must allow for such an approximation in every cell generation (cf. Metz, '16) This would seem to necessitate the assumption that the envelopes of homologous chromosomes could unite.

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EXPLANATION OF FIGURES.

The figures were drawn with the aid of a camera lucida, using a Zeiss 2 mm. oil immersion objective and number 12 ocular. Magnification about 1000 diameters.

All figures except number 4 are from specimen number 2056.

It should be emphasized that the figures represent cross sections at *one level* in most cases, and do not represent entire nuclei.

FIG. 1. Spermatogonial metaphase, probably not entire.

FIGS. 2–10. First spermatocyte growth period.

FIG. 2. Very early growth stage.

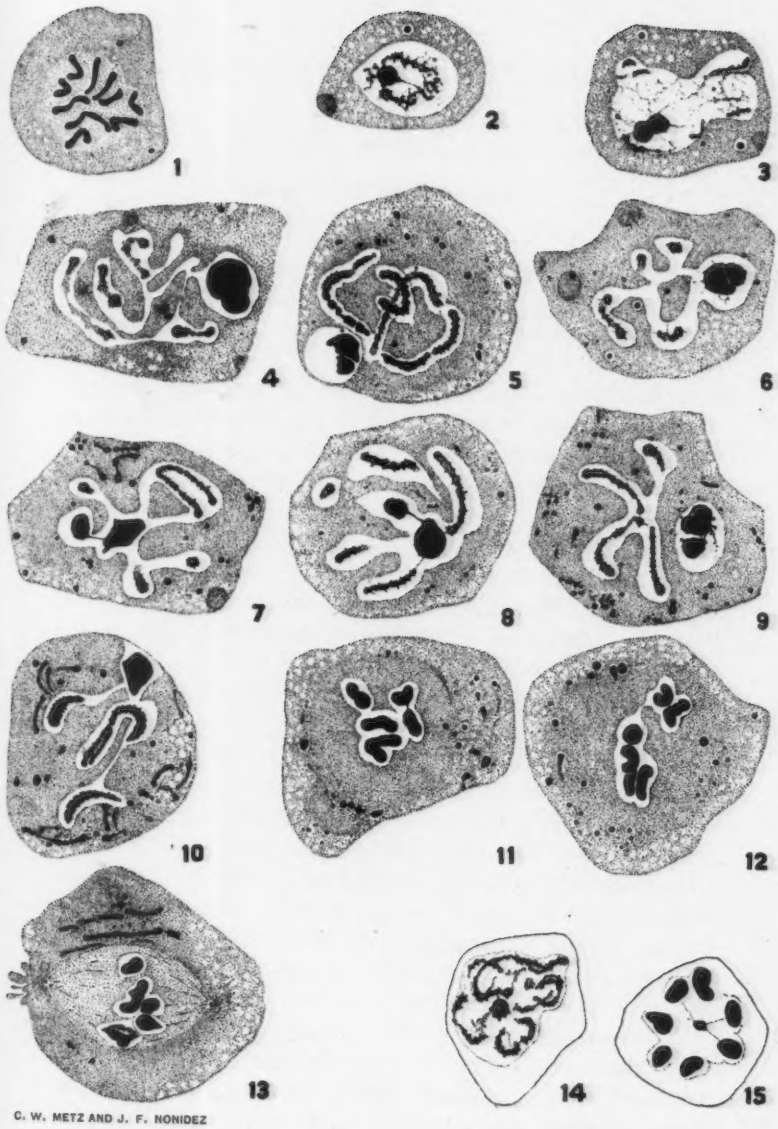
FIG. 3. Slightly later, nucleus becoming lobulated.

FIG. 4. Later, but still in the early part of the growth period; nucleus completely transformed into narrow ramifying chambers.

FIGS. 5–10. Various aspects of the nuclei during the successively later growth stages, showing the lobes and chromosomes cut longitudinally, transversely and at intermediate angles. The isolated portions in figures 8 and 9 are sections of lobes, not separate vesicles.

FIGS. 11–13. First spermatocyte metaphase in polar and lateral views, showing the persistence of the hyaline cortical zone.

FIGS. 14–15. Ptecticus (see text, p. 159).



C. W. METZ AND J. F. NONIDEZ

OF MICH.

THE SUSCEPTIBILITY OF CELLS TO RADIUM RADIATIONS.

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It has frequently been observed that cells may show at different periods of their existence marked variations in susceptibility to radium radiations. An embryonic cell is more quickly injured than the same cell in the adult condition: at the metaphase, cells are more sensitive than they are immediately before or after that brief period (1). Bohn (2) first suggested that the underlying cause of such differences in response must be sought in the physiological condition of the cells at the time of radiation. That they cannot be due to changes in the absorptive power of protoplasm is obvious: whether a cell is sensitive or not, the rays are absorbed to the same extent. The actual changes produced by them in protoplasm must therefore be the same. But the reaction of the cell to such changes differs immensely. We may therefore say that a cell is susceptible when it is in such a physiological condition that a modification produced by the rays results in a greater or less injury, and that it is resistant when the same modification is not followed by injurious effects.

The object of the present paper is to show that among the conditions which affect the susceptibility of cells to radium radiations are (1) the temperature of the cells at the time of exposure, and (2) the relative permeability of the surface layer of the cell.

The experiments to be described were carried out on certain Protozoa, for these cells are better adapted to this kind of experimentation than any others. They can live in both high and low temperatures without injury: and different genera show marked differences in permeability. Cells of the same species, even descendants of the same individual, vary widely in their reaction to radium radiations at different periods of their life cycle. *Paramœcium* is perhaps the best cell for experimental purposes since it is more susceptible than any other common type.

I will not here review the results obtained by other investigators who have tested the effects of radium radiations and X-rays on Protozoa. Different methods of exposing the cells lead to such wide variations in results that comparisons cannot safely be made. In general, however, cells which are sensitive to the one are also sensitive to the other type of radiation. Great differences in the susceptibility of cells of the same species have been reported. Thus Zuelzer (3) states that *Pelomyxa palustris* when exposed to 6 mg. of radium element dies sometimes within one hour and sometimes only after four hours of continuous exposure. Such differences make any conclusion as to the length of the lethal dose out of the question. But with appropriate methods the lethal dose is found to be constant.

Methods.

The method used in the following experiments is this. The radium was enclosed in a glass capsule which prevented the alpha rays from escaping. In the experiments on the relation of temperature and permeability to susceptibility, the strength equalled 13.4 mg. of element. In the third series, on the change in permeability induced by the radiations, the strength was 25 mg. of element. The radium tube was used unscreened and was supported above the drop of culture medium at a distance of 2 mm. Thus all of the rays which emerged from the lower side of the tube could reach the cells. The whole preparation was kept in a moist chamber at the desired temperature.

In order to determine what type of rays produced the effects which are to be described, I interposed between the radium tube and the *Paramœcia* lead sheets of various thicknesses, thus filtering out the more penetrating rays. All of the beta rays are stopped by 2 mm. of lead: the gamma rays are not affected. It became apparent at once that the changes produced in the *Paramœcia* were due to the action of the slowest beta rays, for when a lead screen of 0.12 mm. was interposed the *Paramœcia* were affected hardly at all. This is to be expected, for the surface layer of the cells is very thin and can absorb only those rays which have a low velocity: it offers almost no resistance to rays having considerable powers of penetration.

In conducting these experiments it was found necessary to use

only *Paramœcia* from a pure culture, for unrelated wild cells show great variations in their susceptibility to the rays. Another necessary condition has been mentioned by Jacobs (4), namely, that in each test the same amount of liquid must be used.

The Reaction of Paramœcium to Radium Radiations.

When a *Paramœcium* is exposed to radium radiations under the conditions described, it quickens its movements at first and then gradually slows down and ceases to swim unless the dish is shaken. Later the contractile vacuoles pulsate more and more slowly and finally stop, usually in the expanded condition. If radiation is longer continued, a typical cytolysis ensues. The cells imbibe water, swelling considerably in consequence, and the ectoplasm bulges out in the form of clear vesicles which later run together. Then the pellicle separates from the rest of the cell carrying with it the cilia. The protoplasm is now highly fluid. At this time the macronucleus, in stained preparations, is seen to be divided into several parts. Not infrequently the cells burst violently. These phenomena are in every point similar to those which are observed when *Paramœcium* is treated with a variety of cytolytic agents, as described by Budgett (5), Harvey (6), and Jacobs (4).

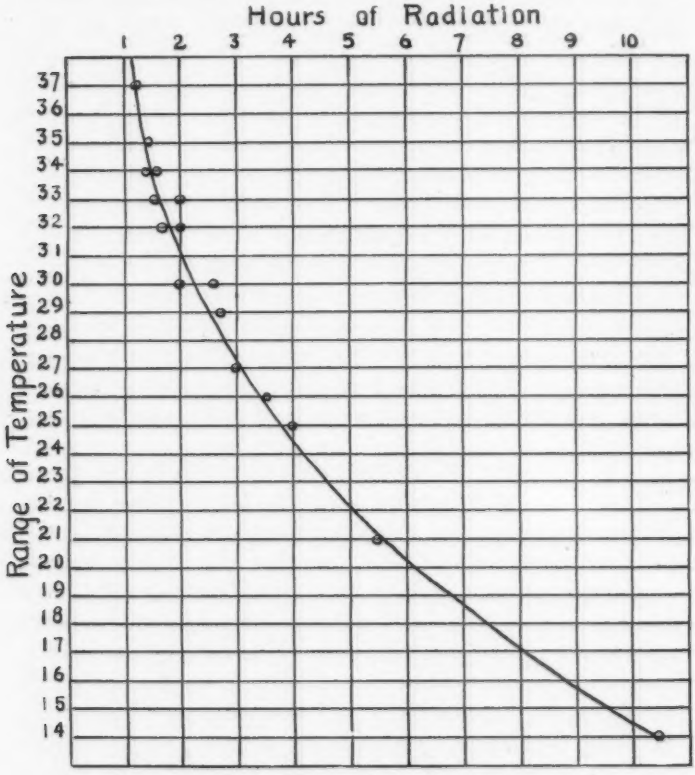
The Relation of Temperature to Susceptibility.

Rhodenburg and Prime (7) first pointed out that there is a definite correlation between temperature and the susceptibility of cells when treated with X-rays. In their experiments they exposed mouse sarcoma in vitro at a temperature of 42° C. to a definite dose of X-rays, and then inoculated healthy mice with the radiated cells. At this temperature 10 per cent. of the inoculations failed to take. When the cells were radiated at 43° C., 76 per cent. of the inoculations failed. Control experiments proved that these temperatures alone are not sufficient to produce this effect. The combination of high but sublethal temperatures with radiation was five times as effective as radiation alone.

Mammalian tissue cannot be subjected to wide variations in temperature, but the Protozoa can live normally at temperatures as low as 15° C. and as high as 37° C. In the following experiments these were the limits employed.

When *Paramœcia* are radiated at high temperatures, they suc-

cumb much more quickly than at low temperatures. The results are shown in the accompanying figure. An analysis of the curve shows that for each increase of about 8° C. the length of the lethal dose is halved. The curve is thus similar to that which expresses the relation between temperature and the velocity of a great number of reactions both inorganic and physiological.



TEXT-FIGURE 1. The effect of temperature on susceptibility.

Snyder (8) cites more than fifty physiological reactions which conform to this type of curve: Woodruff and Baitsell (9) show also that the division rate of *Paramœcium aurelia* varies similarly with changing temperature.

The cells were not injured by these temperatures alone. While

the experiments were in progress the room temperature varied from 30° to 36° C. and the cultures flourished. Single lines of cells showed a steady division rate of about 2½ divisions per day. In the coolest temperatures the cells remained normal, and regained the usual division rate on being brought back into a warmer place.

This increase in susceptibility at high temperatures is not due to any increased activity of the radiations, nor to any change in the power of protoplasm to absorb the rays. The amount of radiation absorbed is determined by the atomic constitution of protoplasm and this does not vary materially during changes in temperature. One of the conditions which varies with the temperature is the permeability of the cell membrane. Hober (10) states that the permeability of plant cells is doubled with each increase of 10° C., the cells being eight times as permeable at 30° as they are at 0° C. That *Paramœcia* are more permeable in warm than in cold solutions can be demonstrated by staining them at 30° and 14° C. in neutral red. In the higher temperature they stain deeply: at the lower, they do not stain at all. Whether a change in permeability is the only cause of increased sensitiveness to radiations remains to be demonstrated. That it is an important factor is shown in the next section.

The Relation of Permeability to Susceptibility.

The fact that cells are most sensitive to radiations when their permeability is increased by heat suggests that the two phenomena are related. Here again the Protozoa are admirably adapted for testing this point, for the permeability of the cell membrane can be measured in the living condition. The method employed in these experiments is that followed by Harvey (6). A number of *Paramœcia* from a pure culture are drawn up into a capillary pipette which is calibrated so that exactly the same amount of liquid is taken in each experiment. The cells are stained for ten minutes in a solution of 0.02 per cent. neutral red mixed with 10 cc. of tap water. At the end of this time the vacuoles at the posterior end of the cells are a bright pink. The surrounding protoplasm is also colored. The neutral red in this dilution is not toxic, although in more concentrated solutions it produces cytolysis.

The cells are now drawn up in a calibrated pipette and added

to exactly 2 cc. of $n/1280$ NH_4OH solution. The amount of liquid thus added reduces the strength of the ammonia to $n/1300$. The stained *Paramœcia* when put into this solution give first the avoiding reaction and quickly begin to lose color. The protoplasm, and later the gastric vacuoles, turn yellow, and finally become colorless. There is a wide variation in the rate at which the color fades in individual cells, some destaining in three or four minutes while others retain some pink color as long as ten minutes. In order to determine the average time for destaining the usual number of cells (about 40), I used the following method. The cells were observed, during their destaining, under a binocular microscope and each one, as soon as it lost color, was removed and the time which had elapsed since its first entrance into the ammonia solution recorded. The following measurements, typical of many, indicate the rate of destaining.

PARAMŒCIUM DESTAINED IN $n/1280$ NH_4OH.

Minutes Elapsed.	No. of Cells Destained.
1	0
2	0
3	0
4	3
5	2
6	4
7	5
8	8
9	6
10	6
11	6
12	5
	Total 45 Ave. 8.5 min.

By this method the personal equation is greatly reduced since the observer cannot form any idea of what the average will be until the entire number of cells has been destained. Many tests on the same pure culture of *Paramœcium* gave very constant results, the average time of destaining in tests carried out on the same day varying less than one half minute. Other lines of *Paramœcia* showed somewhat different averages but even here they differed from each other by not more than one and one half minutes.

A study of *Paramœcium* cells at different phases of their life cycle shows that the permeability varies, being much greater at the time of conjugation than at any other period. Thus among four lines of cells in which conjugation did not occur, the de-

staining time varied from 7.8 minutes to 9 minutes. In a culture which was undergoing an epidemic of conjugation the pairs showed an average destaining time of 4.5 minutes. This culture was presumably not pure, and the pairs in their reaction to the ammonia showed a wider variation than was found in any homogeneous group. But the difference in permeability between conjugants and non-conjugants was in every case large enough to be significant.

Other Protozoa differ from *Paramœcium* in permeability. As Harvey has pointed out, *Stylonichia* and *Oxytricha* are comparatively impermeable. Indeed, after ten minutes in neutral red of the usual concentration they have taken up almost no color at all. The stain must act for twenty-five to thirty-five minutes before these cells are stained sufficiently for experimental purposes, and even after this time they are not as highly colored as *Paramœcium* is after ten minutes. The time required for destaining *Stylonichia* varies somewhat in different cultures, but the average is approximately forty minutes.

When the relative permeability of these cells is compared with the length of their lethal dose of radium radiations, we find a close correlation between the two measurements: that is, cells which are relatively permeable are quickly killed by the rays, while those which are less so are more resistant. The following table indicates these relations.

COMPARISON OF THE DESTAINING TIME AND LETHAL DOSE OF RADIUM RADIATIONS AT 27° C.

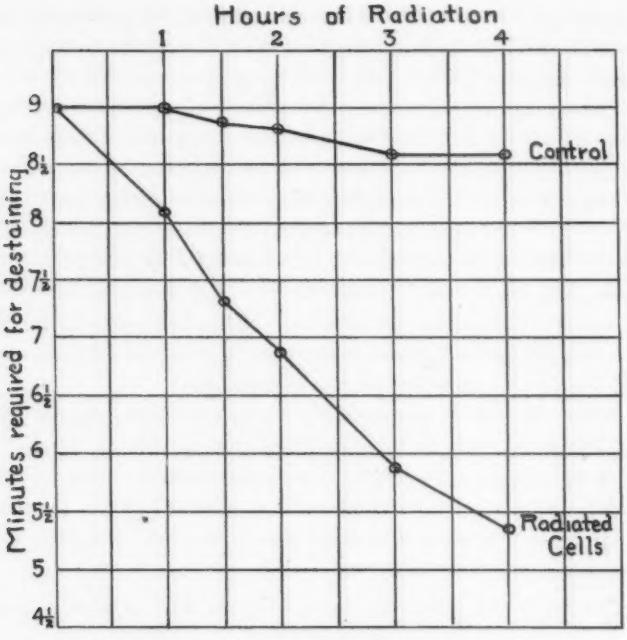
	Destaining Time.	Lethal Dose.
Paramœcium single cells	8.6 min.	3 hours
Paramœcium conjugating	4.5 min.	1½ hours
Stylonichia	40. min.	15 hours

It is evident therefore that the susceptibility of these Protozoa to radium radiations varies directly with the permeability of the surface layer of the cell.

THE EFFECT OF RADIUM RADIATIONS ON THE CELL MEMBRANE.

The question naturally arises, what is the reason for this correlation? The answer is to be found in the fact that the rays which are absorbed produce in the cell membrane changes which lead to increased permeability. The experiments described below indicate the rate at which these changes take place.

In the following experiments the radium amounted to 25 mg. of element. The cells were exposed in the same manner as before, then stained in neutral red and destained in $n/1280$ NH_4OH. The accompanying figure shows the results of experiments per-



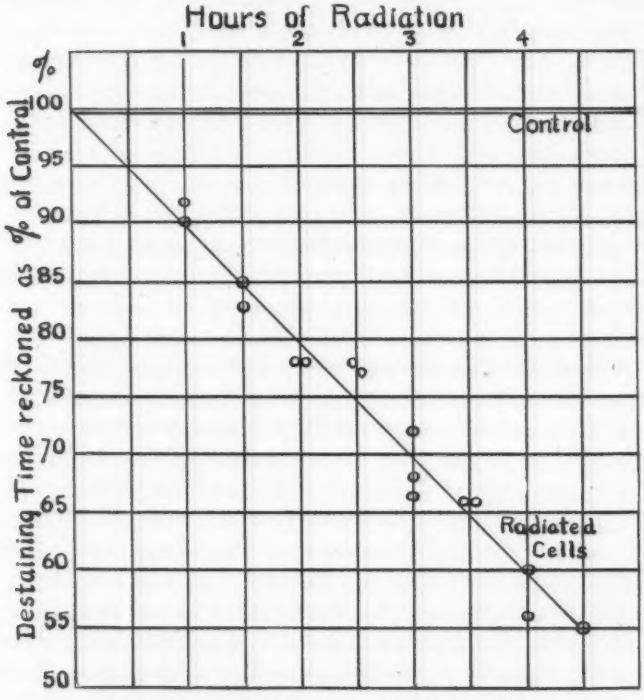
TEXT-FIGURE 2. The effect of radiations on permeability.

formed in one day. It is necessary to make many tests within the limits of a few hours for the cells vary somewhat in their reactions to these manipulations with changing conditions of food, etc. By using two tubes, each amounting to 25 mg. of element it was possible to perform two experiments simultaneously and thus make seven or eight determinations in a single day. The temperature in these tests remained uniformly at 22° C.

The control cells showed a slight increase in permeability after remaining in a small drop of culture medium for some hours. This however is not significant, for cells left as long as eight hours show practically the same reaction to ammonia as those

left for only two hours in a small drop. But the radiated cells display a marked shortening in the time required for destaining, or, in other words, they show a considerable increase in permeability, the change beginning very soon after the exposure to radium commences. At the end of four hours the cells are still alive but are for the most part motionless. If the exposure is longer continued the cells die, showing the characteristic signs of cytolysis.

The second figure shows the result of a larger number of tests.



TEXT-FIGURE 3. The effect of radiation on permeability.

Since they were made during the course of many weeks the controls varied somewhat, although on any one day they were very uniform. The time required for destaining is here given in percentages of the control time which is reckoned as 100 per cent. The curve clearly indicates that the normal semi-permeability of

Paramœcium increases progressively as the exposure is more and more prolonged.

Some of the radiated cells were removed to separate drops of culture medium before treatment with the stain, and their division rate observed. Those which were exposed for one hour or a little less frequently showed a higher rate than the controls. This result has been described by Markowits (11) who studied the effect of mesothorium rays on *Paramœcium*. The acceleration can be observed for five or six generations, after which the cells return to normal and show no evidences of injury. The same phenomenon I have observed in sea urchin eggs when lightly radiated (12). Indeed, a stimulation in the rate of growth is of general occurrence, having been noticed in the case of growing plants, embryos, tissue cultures, and abnormal tissue growths.

From these facts we may conclude that the slowest beta rays increase the permeability of the surface layer of *Paramœcium* cells. If the exposure is brief, this change in permeability is accompanied by an acceleration in the rate of cell division. If it is more prolonged, a destructive cytolysis ensues, and the cells die.

Discussion.

Radiations which are absorbed at the surface of the cell produce definite changes which lead to an increase in permeability, and, if the exposure is sufficiently prolonged, to complete cytolysis. For a definite dose, such changes must be the same in extent, regardless of the physiological condition of the cell, for the absorptive power of protoplasm remains constant. Yet it is quite apparent that a given dose of radiation may result in no appreciable injury in certain instances, while in others it is followed by the death of the cells. This is clearly shown in the text-figures. For example, at 22° C. radiated *Paramœcia* undergo cytolysis, under the conditions described, in five hours. From the beginning of the exposure to the death of the cell there is a steady increase in permeability. An exposure of half this duration is followed by no permanent injury. But when cells are radiated at 30° C. they are cytolyzed after only two and one half hours. Cytolysis occurs when the permeability of the cell has been raised above a definite limit. If it is already high, due to high temperature or to other conditions, the cytolytic action of

the rays quickly raises the permeability above the limit and the cell dies. But if it is low, the lethal point is reached only after a prolonged exposure.

A similar phenomenon is observed in eggs exposed during different phases of mitosis. At the metaphase their permeability, as shown by Lyon (13) and others, is notably greater than at any other period: so also is their susceptibility. I have shown (12) that an amount of radiation which will induce a quickened cell division in sea urchin eggs, when applied just before or after the metaphase, has a retarding effect when applied at that period. That is, the cells are more sensitive then than they are during the prophase or telophase of mitosis.

The same results are obtained when other cytolytic agents are used in place of radium radiations. Lillie (14) finds that if freshly fertilized sea urchin eggs, which are highly impermeable, are treated with hypotonic sea water they resist its cytolytic action for thirty minutes. But if they are placed in this solution when the cleavage furrow appears, they rapidly undergo cytolysis.

It appears possible therefore that the susceptibility of cells may be raised by the simple expedient of increasing their permeability by heat or by some other means. This has been done by Rhodenburg and Prime (7) in the experiments already cited. How far this method can be used in the treatment of abnormal tissue growths remains to be demonstrated.

The fact that agents which differ so widely as do radium radiations and hypotonic sea water produce the same effects under similar conditions suggests that the action of these rays, and also other types of radiant energy, such as ultra-violet light and alpha rays, is not peculiar to themselves. Indeed it may be said that any of the rays which are absorbed at the surface of the cell will cause changes differing in no way from those produced by a great variety of chemical cytolytic agents.

The more penetrating beta rays, the gamma, and X-rays appear to be so slightly absorbed at the surface of cells which are freely exposed to them that they can produce little or no effect. Richards (15) tested the permeability of various eggs and of *Arenicola* larvæ after an exposure to X-rays and found no evidence of any increase. I have shown that the rapid beta and the gamma rays of radium do not act on the surface layer of *Paramœcium*.

But it has been demonstrated that very definite proportions of these rays are actually absorbed by cells of deep-lying tissues. And histological study shows that such cells after radiation imbibe water, swell considerably, and undergo degenerative changes which have all the appearance of a typical cytolysis. It is apparent, then, that any kind of rays which are absorbed produce the same effects. But these more penetrating rays differ from the less penetrating types in this respect, that they are also able to bring about degenerative changes in the interior of the cell, particularly in the nucleus, which the latter, because of their slight penetrating power, are unable to produce.

I have mentioned the fact that a brief radiation with the slow beta rays produces an acceleration in the division rate of *Paramœcium*. This effect is not restricted to the action of these particular rays, for all the radiations of radium, as well as X-rays, can produce this result if the proper exposure is made. This has been demonstrated in a great variety of cases, some of which have already been cited. According to Lillie (16) such acceleration is the direct outcome of increased permeability, for this condition allows a freer interchange of CO_2 and O through the surface layer and a consequent hastening of all metabolic activities. It may be possible, therefore, that all cases of stimulation following exposure to radium radiations and to X-rays can be explained on this basis.

Summary.

1. The susceptibility of *Paramœcium* to radium radiations (chiefly the slowest beta rays) varies with the temperature at the same rate as do physiological reactions of various kinds.

2. The susceptibility also varies directly with the degree of permeability of the surface layer of the cell.

3. The slow beta rays act on the surface layer of the cell, increasing its permeability, and if allowed to act long enough, causing a typical cytolysis. In this respect they resemble other types of radiant energy, and diverse chemical cytolytic agents.

4. Cells which have a relatively high permeability are more susceptible than those having low permeability, for the cytolytic action of the rays is quickly followed in the former by a cytolysis which is irreversible, while in the latter it is reversible.

5. It is suggested that this increase in permeability, following brief exposures, is the cause of the acceleration in division rate seen in *Paramœcium* and in other cells and tissues.

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THE PHYSIOLOGICAL AND SYMBIOTIC RELATIONSHIPS BETWEEN THE INTESTINAL PROTOZOA OF TERMITES AND THEIR HOST, WITH SPECIAL REFERENCE TO *RETICULITERMES FLAVIPES* KOLLAR.*

L. R. CLEVELAND.

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This is the second of a series of papers on this subject. The first paper of the series, "Correlation between the Food and Morphology of Termites and the Presence of Intestinal Protozoa," deals with the more general aspects of the subject, and will appear in the July (1923) number of the *American Journal of Hygiene*.

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INTRODUCTION.

The most unusual and peculiarly interesting faunal association known to parasitology is that represented by the teeming menagerie of intestinal protozoa harbored by termites. These protozoa are favorite objects for study and have attracted the attention of many investigators. The initial microscopic view of thousands of these organisms, but recently removed from the termite's intestine, wriggling, undulating, and twisting in a drop of saline solution, never fails to fill the observer with enthusiasm, and bring forth volleys of questions. Perhaps the first thing to attract attention is the abundance, both in form and number, of protozoa present in a single host. The large abdomen is almost completely filled by the greatly distended intestine, the lumen of which is gorged by a vast horde of protozoa.

The protozoa of the termites, for the most part, differ considerably from those found in other animals, and this difference cannot fail to attract the attention of a protozoölogist. Their most distinctive features are: the ingestion of solid particles of wood for food; the diversity of organization among the various forms; the degree of structural specialization [1] attained by many of the species, resulting in the formation of the most complex flagellate organelles yet known.

No observer has ever failed to raise the question of the relation of the protozoa to their host, but most investigators in the past have regarded the protozoa either as true parasites or commensals, though, in almost every instance, no proof whatever is offered in support of either contention.

At present no termite of the most highly specialized family, the Termitidæ (Metatermitidæ Holmgren), is known to harbor intestinal protozoa; but all the genera and species of the other families, Kalotermitidæ (Protermitidæ Holmgren), Rhinotermitidæ (Mesotermitidæ Holmgren) and Mastotermitidæ, that have been examined [2] have been found to harbor enormous

[1] See especially the papers of Kofoid and Swezy (1919) describing the neuromotor system of *Trichonympha campanula* and *Trichomitus termitidis*.

[2] See the first paper of this series, "Correlation between the Food and Mor-

numbers of protozoa. It is not definitely known why protozoa are never found in the intestine of any of the Termitidæ; but a difference in the feeding habit has been suggested as a very probable explanation, since few, if any, of the Termitidæ feed solely on wood.

THE PROBLEM.

To determine the relation of the intestinal protozoa of termites to their host.

Experimental methods were the paramount mode of attack; and it was necessary, in order to solve the large problem stated above, to solve several problems, which may be briefly stated as follows:

1. What is the principal compound in wood, the sole article of diet of most termites, which is used for food.

2. How do termites utilize cellulose, the principal compound in wood, as their chief article of food?

3. How may all of the intestinal protozoa be killed without at the same time injuring their host? To develop a rapid and successful method for removing the protozoa from the termites was a very difficult proposition.

4. Why do termites, when subjected to a temperature of 36° C. for 24 hours, lose their ability to maintain themselves on a wood diet?

5. Is defaunation (the removal of the protozoan fauna), which occurs when termites are incubated at 36° C. for 24 hours, in any way responsible for the inability of the termites to maintain themselves on a wood diet?

6. Why do the incubated and defaunated termites live indefinitely when fed the decomposition products of wood, or the products of fungus-digested cellulose; but die within 10–20 days when fed wood, their normal diet, or when fed cellulose?

7. Why do the incubated and defaunated termites regain their ability to make use of wood or pure cellulose as food when reinfected (refaunated) with protozoa?

8. Do the unincubated and faunated termites harbor any in-

phology of Termites and the Presence of Intestinal Protozoa," for a list of the genera and species that have been examined. Since writing this more than 75 species of the Termitidæ have been examined and three of this number have been found to harbor protozoa and feed on wood.

testinal bacteria or fungi, capable of digesting cellulose, which are killed off during incubation?

9. Do the protozoa aid their host mechanically to digest wood, or do the protozoa digest the wood particles which they take into their bodies and convert them into substances which their host uses for food?

10. Since the termites lose their ability to digest wood when the protozoa are removed from their intestine, that is when they are defaunated, but regain this ability when reinfected with protozoa, and since the protozoa themselves can be shown to digest the wood particles which they take into their bodies, does a true symbiotic relationship exist between termites and their intestinal protozoa?

MATERIAL.

Reticulitermes flavipes Kollar, collected in Maryland, near Baltimore, was used for all the experimental work on this genus, though many colonies from other states have been used for studying the protozoa of this species. *Termopsis* sp. (the species could not be determined because there were no winged adults in any of the colonies collected, but it is either *angusticollis* Hagen or *nevadensis* Hagen) from Ashland, Oregon, owing to its large size, was used in many of the feeding experiments, though *Reticulitermes flavipes* was also used in all of the feeding experiments. *Kalotermes schwarzi* Banks, *K. jouteli* Banks and *Prorhinotermes simplex* Hagen, from southern Florida, were used for incubation experiments, but the material of these genera was not sufficient to make possible as extensive a study as was carried out with the material from Maryland. *Amitermes* sp., from Uvalde, Texas, and *Nasutitermes morio* Latreille, from Porto Rico, both of the family Termitidæ, were studied.

ACKNOWLEDGMENTS.

Many thanks are due Drs. R. W. Hegner and W. H. Taliaferro, of the Department of Medical Zoölogy, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland, for valuable suggestions and criticisms. I am also very deeply indebted to Dr. T. E. Snyder, Specialist in Forest Insects, Bureau of Entomology, U. S. Department of Agriculture, Washington, D. C., who identified all the termites studied and furnished all the material except *Reticulitermes flavipes* and *Nasutitermes morio*.

GENERAL CONSIDERATIONS.

1. The Termites.

a. Castes.

In order that the reader, unfamiliar with the nature of a termite colony, may know something of the castes present, the following condensed statement (taken from the papers of Snyder and Thompson) is made. In *Reticulitermes flavipes* there are five castes, three of which are fertile or reproductive, and two that are sterile or non-reproductive. Males and females occur in each of the five castes. The reproductive castes are: (1) the *first form*, which has three well-defined phases of development: (*a*) the nymphs, with long wing pads, creamy white body 1.3–1.4 mm. long, and light brown eyes; (*b*) the winged adults, with long wings, dark brown body 6 mm. long, and black eyes; (*c*) the older males and females, with enlarged abdomens and the scales of the shed wings, body 7–14 mm. long: (2) the *second form*, which, like the first form, has three well defined phases of development: (*a*) the nymphs, with short wing pads and colorless body and eyes; (*b*) the young adults, with short scaly wing vestiges, straw-colored or grayish body 6–7 mm. long; (*c*) the older adults, with wing vestiges, enlarged abdomen, body length 7–12 mm: (3) the *third form*, which also has three developmental phases: (*a*) the nymphs, wingless, with white head and body, and eyes that are invisible in the living or unstained specimen; (*b*) the young adults, wingless, head and body pure white, opaque and not transparent, about 6 mm. long; (*c*) the older adults, with enlarged abdomen, wingless, head and body white, 7–9 mm. long. The other castes, the sterile castes, are: (4) the *worker*, wingless with grayish abdomen, only two developmental phases, *i.e.*, nymphs and adults, always feeds on wood and there is no degeneration of the jaw muscles, always harbors protozoa once an infestation has been acquired, salivary glands small and very little fatty tissue is present in the body; (5) *soldier*, wingless with elongated head covered with thick yellowish chitin, mandibles dark brown long slender and curved, abdomen shorter than in other castes and more flattened, nymphs and adults only, no post adult growth.

Thompson (1917) showed that the newly hatched nymphs of

Reticulitermes flavipes, 1.1 mm. long, although externally all alike, could be differentiated by their internal structures into two distinct types, namely, (*a*) reproductive nymphs, from which develop the three fertile adult castes, and (*b*) the worker-soldier nymphs, from which develop the two sterile adult castes. By the time the reproductive nymphs had attained a body length of 1.3–1.4 mm. they could be differentiated by their internal characters into nymphs of the first form and nymphs of the second form, which developed, finally, into the two respective reproductive adult castes. Soldier and worker nymphs could be differentiated, internally, when they had attained a body length of 3.75 mm. Nymphs of the third form could not be differentiated until a body length of 4 mm. was attained.

Several intermediate forms have been discovered by various investigators, intermediates between the first and second forms, three fertile soldiers (Heath, 1902)[1], and others; but these need not be considered here.

The five castes occur in most termites. Known exceptions are as follows: the third form reproductive caste occurs in few, if any, of the Termitidæ; in fact none have been reported from the Nearctic genera of this family, and it is doubtful if this caste occurs in any genus of the Termitidæ. Apparently this caste has been lost in this, the most highly specialized, family of termites. The third form is less common than the first and second in *Reticulitermes flavipes*, but in *Termopsis* it is more common than the first and second form. *Reticulitermes* is about midway between the most primitive and the most highly specialized termites. *Termopsis* is a primitive genus. A true worker caste is not present in *Termopsis* and *Neotermes*, but a large-headed worker-like reproductive form is present. Two genera, *Kalotermes* and *Cryptotermes*, have no worker caste. The genus *Anoplotermes* has no soldier caste.

Grassi calls the first forms "true" or "perfect insects," or

[1] Wheeler (Social Life among the Insects, Scien. Monthly, 16, 1923, p. 172) says: "The soldiers and workers are normally sterile, but sometimes they become fertile and probably on such occasions reproduce their own castes." Snyder (personal communication) soys Heath now admits he was wrong. Also experiments which I have conducted recently reveal the fact that the soldiers of at least several, and probably all, species of xylophagus termites cannot live to themselves when given their normal diet of wood.

"the royal forms." The second and third forms he calls "substitute" and "complemental" forms, which forms he thinks are always ready to take the place of the royal forms in case of need. He recognizes nymphs of the first and second forms, but these, he thinks, if taken young enough and given the proper food, may be changed into anything else, *i.e.*, any of the castes. But, Thompson (1919 and 1922) and Snyder (1920) have presented valuable experimental evidence in support of the view that each of the three reproductive castes breeds true, that is, produces reproductive adults only like itself.[1]

b. Food—Direct.

Wood is a direct food of most termites. Some of them feed on dry heartwood, while others feed on moist and more decayed wood, usually sapwood. *Kalotermes schwarzi*, for instance, is often found in furniture stored in an attic, since it does not require access to the soil in nature as does *Reticulitermes flavipes*. However, it is quite probable that *R. flavipes* requires access to the soil only in order to obtain moisture, since it may be kept in captivity indefinitely without access to soil provided sufficient moisture is given it. Water, of course, is a direct food, though some termites require very little of it. *Amitermes tubiformans* Buckley, for example, feeds principally on dry cow chips and soil, but occasionally eats the roots of grass and other vegetation. The manure is often carried down into chambers below the ground. Many of the Termitidæ feed on plants, sometimes attacking flowers, shrubs and field crops. Fungus gardens are cultivated by many speices of this family. These termites carry large quantities of vegetation into their huge nests and take advantage of the digestive action of the fungus on the hemicellulose, the fungus and hemicellulose being eaten by them as food. It is interesting to note that leaf-cutting ants (*Œcodoma*) do the same thing. They carry thousands of leaves to their nest, where they use them to make a compost heap, on which fungi, to which the ants are very partial, grow. Termites in captivity may be induced to eat almost anything, but our knowledge regarding

[1] Each of the adult sexual forms can reproduce itself and the forms below it in rank. Thus, the first form adults reproduce all castes: the second form individuals like themselves, third form, soldiers and workers; the third form individuals like themselves, soldiers and workers.

their habits in nature, especially the Termitidæ, is so limited that it is impossible to adequately discuss their food.

c. Food—Indirect.

The liquid diet is either a salivary secretion or regurgitated material from the mid-gut. It is very probably the former. It is a colorless and distinctly alkaline liquid which collects on the labrum as a small drop, which may be used either as food for others or for the termite secreting it. Many termites also employ this liquid in building. In *Reticulitermes flavipes* the young larvæ of all the castes and the second and third forms during the third developmental stage feed solely on this liquid diet which they receive from other members of the colony. Later, the larvæ of all the castes eat the excreta as it is passed from the anus of the older members of the colony, and it is at this time and in this way that they first become infected with protozoa. The larvæ also begin to eat wood at or about the same time that they begin to eat excrement—which in this termite is composed of a few wood particles, but primarily of protozoa and liquid. The soldiers, workers and first form reproductive males and females always feed on wood, though they—especially the first forms during the third developmental stage—partake of a liquid diet as well. The exuviæ are usually eaten and, sometimes, the dead bodies of other members of the colony are eaten.

2. THE PROTOZOA.

Lespes (1856) made the first recorded observation of termite protozoa, though he did not describe them. Leidy (1877 and 1881) was the first in America and the second in the world to observe these organisms. In 1881 he gave descriptions of the protozoa which he had observed in *Reticulitermes flavipes*, creating three genera for them, *Trichonympha*, *Pyrsonympha* and *Dinenympha*. This termite really harbors several other genera, most of which were included in Leidy's drawings as stages in the life cycle of the three genera mentioned above. More than a score of other investigators have studied these protozoa, and to date 41 genera have been described. For a complete account of the work done by all the students of termite protozoa and for a classification of all the protozoa which have been described from

termites the reader is referred to the first article of this series, "Correlation between the Food and Morphology of Termites and the Presence of Intestinal Protozoa."

All of the known termite protozoa, except *Gregarina termitis* and *Nyctotherus termitis*, have been placed in two orders of flagellates, the *Polymastigina* and the *Hypermastigina*, the latter being the most highly specialized order of flagellate protozoa.

3. Biochemical Considerations.

Dore (1920) gives the following analysis of yellow pine (*Pinus ponderosa*) oven-dry (100° C.) basis. Results in percentages.

Benzene extract	2.22	Lignin	29.47
Alcohol extract	1.49	Xylan	3.49
Cellulose	57.72	Mannan	6.37
		Galactan	0.78

The two following examples are taken from Ritter and Fleck (1922), who have analyzed a large number of American woods:

Analysis of a mean of four samples. Results expressed in percentages of oven-dry (105° C.) material.

	Western Yellow Pine (*Pinus ponderosa*).	Tanbark Oak (*Quercus densiflora*).
Moisture	6.42	3.66
Ash	0.46	0.83
Solubility in cold water	4.09	4.10
Solubility in hot water	5.05	5.60
Solubility in ether	8.52	0.80
Solubility in 1% NaOH	20.30	23.96
Acetic acid	1.09	5.23
Methoxy	4.49	5.74
Pentosan	7.35	19.59
Methyl pentosan	1.62	none
Lignin	26.65	24.85
Cellulose	57.41	58.03
In the cellulose:		
Pentosan	6.82	22.82
Methyl pentosan	1.98	none
Alpha-cellulose	62.10	56.77
Beta-cellulose	10.56	19.92
Gamma-cellulose	30.13	23.93

That the principal food of most termites is cellulose is a fact which appears to have escaped the attention of physiologists and biochemists. Because of this, and also owing to the fact that our

text-books of chemistry and physiology give little, if any, of the information which has been gained in recent researches on the chemistry and digestion of cellulose, it seems expedient to briefly mention—with no pretention at a discussion—some of the salient features brought out by a few of the more important of these researches.

According to Hibbert (1921) "cellulose is nothing more than a polmerized dextrose glucoside of dextrose," and Irvin and Hirst (1922) in their most recent paper on the constitution of polysaccharides have shown this to be true. They state that "the average yield from the polysaccharide (*e.g.*, cellulose) to the hexose (*e.g.*, glucose) is thus 95.5 per cent. of the theoretical amount. Considering the standard of purity in which the mixed methylglucosides were isolated, there can be no further doubt that cotton cellulose is composed entirely of glucose residues."

Pringsheim (1912) observed cellobiose—a disaccharide which bears the same relation to cellulose that maltose does to starch and which spilts into two molecules of glucose (Fischer and Zemplen, 1904 and 1910), when acted upon by cellobiase—as an intermediate product of bacterial cellulose digestion. The exact mode of linking together of the two glucose residues in cellobiose was determined by Haworth and Hirst (1921). Groenewege (1920) showed that the bacterial digestion of cellulose is a hydrolytic process.

Cellulase is known to be produced by various moulds, soil fungi, pathogenic fungi, actinomycetes, soil and intestinal bacteria. Cellase is to be distinguished from cellulase in that it acts on the hemicelluloses and is not an endo-enzyme (Pringsheim, 1912).

Some rather divergent views have been expressed regarding the products of cellulose digestion; different investigators working with different cellulose-decomposing organisms have gotten different results. But according to Cross and Dorèe (1922) most of these differences may be accounted for. The typical result of the digestion of wood cellulose is about as follows: Cellobiose, glucose, acetic acid, lactic acid, butyric acid, alcohol, CO_2, H_2, CH_4, CO. Under excessive aëration alcohol and acetic acid are completely oxidized to CO_2 and H_2O. The gas liberated is never pure CO_2; there is either H_2 or CH_4, or a mixture of the two. Alcohol is never obtained without acetic acid. Waksman (1922)

is of the opinion that some soil bacteria cause CH_4 fermentation of cellulose and that others cause H_2 fermentation. McBeth (1913) thinks cellulose-fermenting organisms are not responsible for the production of gases. Gas, he thinks, is produced by contaminating organisms.

Abstracting Klason's remarkable researches on lignin, Cross and Doree (1922) state: "*Beta*-lignin (C_{19} H_{18} O_9, and that part of lignin precipitated by arylamine bases is designated as acrolein-lignin or *alpha*-lignin. The other part, which is not precipitated, appears to be a carboxyl group and is called carboxyl lignin or *beta*-lignin) must therefore be bound to cellulose and probably to *alpha*-lignin ($C_{22}H_{22}O_7$). Lignin cannot well be assumed as a secondary product derived from cellulose, but appears as a direct assimilation product of CO_2 and H_2O or formaldehyde. Therefore the formation of lignin is a function of chlorophyll." Klason thinks lignin is possibly present in wood as a glucoside and that it may be built up from pentose.

THE RELATION OF THE PROTOZOA TO THEIR HOST.

A. Historical.

1. *True Parasites.*

Most students of termites, and termite protozoa, have focused their attention on the morphology[1] and behavior of either the termites or the protozoa which they harbor; consequently, the interrelation of host and parasite has been very little investigated.

Grassi is really the only investigator who has not confined his attention almost exclusively to a study of either the termites or their intestinal protozoa. He has studied the termites and the protozoa, but has never carefully investigated their relationship. His work on the two groups of organisms, termites and termite protozoa, has been largely a study of two separate and distinct problems; namely, the morphology and systematics of the protozoa, and the physiology and behavior of the termites, with special emphasis on the underlying factors in caste production.

Grassi and Sandias (1893) think that the relative abundance of protozoa present in the various adult individuals in a termite

[1] The morphology of termite protozoa is a fascinating subject and occupied the writer's entire attention for a year before the present investigation began. He will publish in a later paper a description of several new forms.

colony is inversely proportional to the degree of gonad development of their host. According to these investigators, the protozoa, with a small amount of saliva, function in the development of soldiers and workers. Why the same thing (many protozoa and a small amount of saliva) functions in the production of two separate and distinct castes, they do not consider. They observed that the protozoa were scarce in the winged sexual forms, and usually totally absent in the neoteinic [1] individuals. When they found protozoa abundant in the neoteinic individuals, the gonad development was imperfect. They remarked that the developing neoteinic forms were fed largely on a salivary diet, which they thought killed the protozoa, though it is more probable that the protozoa died because their host no longer furnished them with wood for food. Then, according to Grassi and Sandias, it is impossible to say whether the maturation of the gonads is due solely to the change from a wood to a salivary diet or to the absence of protozoa. In this paper they reach no definite conclusion, though they do not believe the presence or absence of protozoa a sufficient cause to control gonad development, for they say: "It is a moot point whether the maturation of the generative organs is due solely to the saliva or to the absence of protozoa as well; but the latter, as my [2] previous remarks show, is not by itself a sufficient cause." Then in the next paragraph," I have frequently asked myself whether the protozoa have not an important digestive function, since the comminuted wood passes almost entirely through their bodies. It is probable, but not proved."

Brunelli (1905) followed up the idea of Grassi and Sandias, and states that in the queens of two genera harboring protozoa, *Kalotermes flavicollis* and *Reticulitermes lucifugus*, there is a correlated destruction of the oöcytes—a kind of indirect parasitic

[1] The term neoteinia was first introduced by Camerano (*Bull. Soc. Ent. Ital*,. 1885, pp. 89–94) to note the persistence during adult life of part or all of the characteristics normally peculiar to the immature, growing, or larval stages. Grassi and Sandias probably refer to the second and third form reproductive castes since it has been shown by Thompson (1920) that the jaw muscles of these forms degenerate to the extent that they cannot eat wood and must be fed on a fluid diet; and, of course, we should not expect them to harbor intestinal protozoa when they do not eat wood.

[2] Grassi wrote most of the paper. The part written by Sandias is placed between asterisks.

castration ("castration parasitaire"). Feutand (1912) says what Brunelli regards as signs of degeneration is in reality nothing more than an alteration of tissue brought about by the histological reagents.

Grassi, nineteen years later (1911), collaborating with Foa, is of the opinion that the protozoa do not aid their host in the digestion of food, and he points out that many wood feeding insects do not harbor protozoa. He states[1] that several workers (species not mentioned) of *Kalotermes*[2] were placed in small boxes with wood and incubated at about (*circa*) 35° C. It is interesting to note that none of the protozoa harbored by *Kalotermes schwarzi* Banks, from Miami Beach, Florida, are killed when incubated for three days at 35° C. How long Grassi's species of *Kalotermes* was incubated is not known, for he does not mention this in his paper. But he does state that the incubation killed off nearly all the protozoa, all of the large forms being killed off every time. These termites, he states, lived in full activity for several months.

Recently Jucci (1920 and 1921), in a preliminary paper reported before the Academy of Lincei, has announced the discovery of a particular diet which brings about caste production. But the carefully controlled breeding experiments and the numerous field observations of Thompson and Snyder (1919, 1920, etc.) make it highly probable that at least three of the castes, namely, the first, the second and the third form reproductive males and females, only give rise to fertile reproductive males and females like themselves and to infertile males and females of the soldier and worker castes.

2. *Commensals.*

If these entozoic protozoa benefit from their association with the termites, and by so doing do not harm or benefit their host, they are commensals. They may feed from the waste food-

[1] Per mio conto in questi ultimi anni ho cercato di decidere la questione in altro modo: se si tengono ad una temperatura di circa 35° C. scatolette contenenti legno pieno di *Calotermes*, i Protozoi muoiono, talvolta tutti, più spesso restano in vita soltanto le forme piccole; si hanno così delle colonie di *Calotermes* senza *Joenia* e *Mesojoenia*, e talvolta con molti individui anche del tutto privi di Protozoi. Io le tengo in vita prospera da parecchi mesi; perciò ritengo che i *Calotermes* possano digerire il legno anche senza gli speciali Protozoi (*Joenia* e *Mesojoenia*).

[2] Also spelled *Calotermes*

products and bacteria in the termite's intestine, or they may be food-robbers and feed from the same table as their host; in either case, they are commensals, provided the host's table always contains sufficient food for both host and parasite. Kofoid and Swezy (1919) are the principal exponents of the commensalistic relationship of termite protozoa. They remark: "One of the most curious and unique faunal associations to be found among the parasitic [1] protozoa is the group parasitic [1] or commensal in the intestinal tract of the social termites. . . . This distension is caused by the vast numbers of parasitic and commensal protozoans which fill the lumen of the intestine. When this is opened a thick milky fluid exudes. Under the lens this is found to be composed of great quantities of these small forms, thickly massed together, along with fragments of wood upon which the host, as well as some of the commensals, feeds."

3. *Symbionts.*

Imms (1919) in studying the termite *Archotermopsis wroughtoni* Desneux made sections of twelve worker-like females and found numerous protozoa and nearly full sized ova ready for fertilization, and in no instance did he observe even an indication of oöcyte degeneration. He also found well developed ovaries in five soldiers, whose hind-intestines were filled with protozoa. Imms suggests that the presence of protozoa may be correlated with the wood feeding habit of the termites. In the young larvæ, and in the queens and kings, which receive the fluid diet from the other members of the colony, he found, as a rule, no protozoa. In the older larvæ, soldiers, workers, and sometimes in the winged sexual forms,[2] protozoa are abundant and a wood diet is the rule.

[1] This term, loosely used, means any organism which lives in or on another organism.

[2] It is interesting here to note the statement of Snyder (1920), regarding one of his cross-breeding experiments. He says, "A large series of young second form female adults of *Reticulitermes flavipes*, which possibly may have been fertilized by second form males, were taken from a fairly small colony and placed with mature first form dealated males, which had not copulated, in small shallow cells in decayed wood sunken in moist sand in glass jars and tin boxes.

After a period of ten days to two weeks all these second form females had died, but the first form males were still living and were active; they evidently were prepared to forage for themselves, whereas the second form adults needed the care and nourishment usually afforded them by the workers. Possibly the jaw muscles

The wood, according to Imms, after being crushed by the mandibles and later by the gizzard, passes rapidly to the hind-gut without undergoing any chemical change. The pyloric valve prevents the readmission of wood particles into the mid-gut. To quote Imms: "On reaching the hind-intestine the wood is in a condition of minute fragments and particles, and the greater bulk of it gradually becomes taken up and absorbed by the numerous intestinal protozoa. Within the protoplasm of these organisms the woody material undergoes chemical changes, and, when ejected from the bodies of the protozoa, much of it is in a condition capable of being assimilated as food by the host termite. A significant fact is that lignous particles are not subjected to the action of the secretions of the mid-gut."

Imms further points out that, in his opinion, the experiments of Grassi and Foa (1911), where these authors claim to have kept *Kalotermes* (species not given, nor is the number of termites) alive and active for several months with all but a few of the intestinal protozoa killed off by incubation at about 35° C., has no value, for during incubation the termites very probably subsisted on the large amount of fecal matter [1] which had accumulated in the jars where they had been kept previous to incubation.

Imms, then, through an ingenious method of reasoning, reaches the conclusion that the protozoa are symbionts, a conception already reached by Buscalioni and Comes (1910), who claim, by means of various microchemical tests, to have shown that the wood is digested inside the bodies of the protozoa; and since the protozoa digest the wood, these authors conclude, that they are symbionts. [2] They state that *Trichonympha agilis*, harbored by *Reticulitermes lucifugus*, when treated with iodine dissolved in iodide of potassium, gives a characteristic glycogenic reaction in a region near the nucleus, and that this reacting region is sharply

in these young second form adults had begun to degenerate through disuse in masticating wood. It is believed that second form males—like the females—would have succumbed, without the workers." First form adults, so far as my experience goes, harbor protozoa, but the second forms do not. This probably explains the inability of the second form adults to live without workers, that is in the event the jaw muscles permitted them to eat wood. Of course the protozoa are lost, possibly, because the host does not eat wood. I have experiments started which I hope will determine this point.

[1] The fecal matter contains sugars and possibly other foods.

[2] A discussion of this may be found on pages 193–4.

defined from the rest of the body. Cutler (1921) could not locate a definite glycogen reacting area in *Pseudotrichonympha pristina*, harbored by *Archotermopsis wroughtoni*, but, on the contrary, found that the glycogenic reaction was diffused through the entire organism. Grassi and Foa (1911) state that they in part attempted to verify the work of Buscalioni and Comes but that their results did not justify the drawing of any conclusions.

Dobell (1921) says: "When the association benefits both parties, the condition is one of symbiosis—a not very frequent state in nature. An example is afforded by some of the flagellates living in termites. In return for the food and lodging which the termite gives to the flagellate, the latter helps the former digest its own food." Regarding this statement Dobell (1922)[1] says: "It is merely a general statement of my own personal view. It is not based on any particular experiments which I have made, but is an inference from what I myself have observed and others have recorded. For many reasons I consider it probable that the flagellates of termites aid their host in digesting the wood on which they feed. The observations of Buscalioni and Comes (1910) and of Imms (1919) especially seem to me to justify such a conclusion.

"However I am quite ready to admit that the matter has not been definitely settled beyond all question. In fact I recently discussed the problem with Dr. Koidzumi and we planned several experiments to determine the question. . . ."

B. Original Observations.

At best some of the experiments of Buscalioni and Comes (1910) were conducted in a rather crude manner and, in addition to this, unwarranted conclusions were drawn due, often, to misinterpretation of the results obtained. The only conclusion which may be drawn from the results of their experiments is, that the protozoa digest the wood which they take into their bodies. But the fact that the protozoa digest the wood in no way hinders the termite from doing the same thing. Many animals ingest more food than they can use. The protozoa may feed from the wood particles in the termite's intestine, as one would naturally expect them to do, since nutrition with most of them appears to be holozoic.

[1] Personal communication.

In order to establish the fact that the protozoa are symbionts, rather than commensals (feeding from the same table as their host), it must be shown that they actually aid their host in some way by doing something for the host which the host itself cannot do. The experiments of Buscalioni and Comes do not in any way fulfill this condition. They have only shown that the protozoa digest wood particles. The ability or inability of the termites to do the same thing was not studied. That the termites aid the protozoa, by furnishing them food and lodging, is obviously demonstrated by the fact that the protozoa, so far as known, cannot live outside of termites. But we cannot call the protozoa symbionts, until their ability to digest wood has been shown to be a necessary aid to the termite.

Realizing the difficulty of determining the relation of the protozoa to the termites so long as the two were associated together, several experiments were begun, with the hope of effecting a method for removing the protozoa from their host, and at the same time leave the host uninjured. It is needless to say that hundreds of experiments—some of which are given in Table I.—met with failure, for the host was either killed or severely injured in an effort to remove the protozoa from it.

When wood, previously soaked in a 5 per cent. aqueous solution of either sodium, potassium or calcium chloride, was fed to a colony of *Reticulitermes flavipes* (elsewhere in this paper—unless it is otherwise evident—when the words "termite," "termites" and "host" are used they refer particularly to this termite) harboring protozoa, within four to five days an occasional termite, free, or almost free, of protozoa was found, when many individuals from the colony were examined. The number free of protozoa increased as time progressed but, before all the termites in the colony had lost their protozoa, it was quite evident that the termites themselves were not normal. They had in some way been affected; whether by a direct action of the chemicals or by a gradual loss of the protozoa, it is impossible to say from the data available.

About this time a much more promising method for removing the protozoa was evolved; namely, the incubation method, and the experiments employing the use of salts, etc. (see Table I.), to remove the protozoa, were discontinued. It might be well to

mention here that, because of the success of the incubation method, many of the other attempts to free termites of their intestinal protozoa were, perhaps, not given a fair trial. If different dilutions of some of the chemicals had been used, the results, possibly, would have been more promising. Had X-ray and ultra-violet light been used more extensively, excellent results might have been obtained.

It was found that all—not one was left alive—of the protozoa could be removed from the intestine of *Reticulitermes flavipes* by incubating them for 24 hours at 36° C. This was a much more rapid method than the feeding of salts, as described above, where several days are required to remove the protozoa from all the individuals in a colony and, besides, the termites after incubation seemed to be perfectly normal in every way. All other attempts to remove the protozoa were now abandoned in favor of the incubation method, which may be briefly described as follows: Sometimes stumps and large pieces of wood, containing thousands of termites, were brought to the laboratory without molesting or disturbing the colony. The stumps and large pieces of wood were placed in glass jars and incubated for 24 hours at 36° C. At the end of the incubation period the jars were taken from the incubators and, after many termites from each jar had been carefully examined to be certain that the protozoa had all been killed, were left at room temperature, which was usually about 20°C. Sometimes the stumps and logs were split before they were brought to the laboratory and the termites were taken from them and placed, with a good quantity of wood, in glass jars with metal tops. These jars were placed in the incubator as soon as they reached the laboratory. In other experiments, where termites were counted, small shell vials with cork stoppers were used, and from 10–100 termites were placed in each vial, and incubated in the same way that those in the large jars were. Several vials of termites without wood, and several with pure cellulose [1] (Whatman's filter paper) only, were incubated. Other experiments in incubation were carried out with results as shown in Table I. One very significant fact brought out by these ex-

[1] This paper was made by W. & R. Balston, Ltd., and contains .0004 grams of ash per circle of 55 mm. diameter. When "pure cellulose" occurs in this paper it refers to this grade of the genuine Whatman filter paper.

periments is that the thermal death point of the protozoa is 3–4° C. lower than that of the insect host.[1]

After incubation the termites were closely observed to note if they at any time became abnormal; to prevent the growth of moulds in the jars, for moulds are very destructive to termites; to be certain that the jars contained neither too much nor too little moisture—a thing that can only be learned from long experience in keeping large numbers of termites in captivity and an intimate knowledge of their habits in nature. Every known precaution was used to keep the termites during incubation in exactly the same environment, except temperature, as the unincubated controls. After incubation the incubated and the unincubated termites were kept under identical conditions, as regards food, temperature, moisture, light, etc.

The termites become abnormal in from five to fifteen days after they have been removed from the incubator. They are less active and their abdomens, if carefully examined, may be seen to be smaller and slightly flattened. The length of time required for abnormalities to appear depends no the kind of wood fed after incubation, the more decayed the wood the longer it is before any abnormalities appear. In two to five days after this first symptom is noticed the abdomen becomes still more flattened. Soon it is very much smaller and almost flat, and two or three days later the animal can scarcely be made to move at all, for only a modicum of vitality remains. Death occurs, on the average, from 10–20 days after incubation. It occurs, as shown in Table II., in some instances in less than ten days and in a few cases after twenty days, the longest being 27 days. In the less-decayed wood, as mentioned above, death occurred early, and in the most decayed, it occurred late. But why did it occur at all? Why did the incubated termites die?

The death of the incubated termites—sterile, uninfected, uninfested and defaunated, so far as protozoa are concerned—may be due to the change in temperature, *i.e.*, the incubation *per se*; it may be due to the killing off of all the intestinal protozoa; or it may be due to the killing of intestinal bacteria and

[1] *Termopsis* sp. from Ashland, Oregon, is killed at a lower temperature. It is killed in less than 24 hours at a temperature of 35.5 degrees C. Work on the T. D. P. of termites from various parts of the world is now in progress and the results will be published in a later paper.

fungi, which, possibly, in some way aid their host in the digestion of wood and pure cellulose.

TABLE I.

METHODS AND RESULTS OF VARIOUS ATTEMPTS TO FREE TERMITES OF THEIR INTESTINAL PROTOZOA.

Method.	Number of Experiments Carried Out.	Approximate Number of Termites Used in Each Experiment.	Results.
Wood,[1] 5% NaCl ..	30	500	Kills protozoa in 10–15 days, but host is almost dead by this time
Wood,[1] 5% $CaCl_2$ ..	10	100	Same result as with NaCl
Wood,[1] 5% KCl....	20	500	Same result as with NaCl
Wood,[1] $HgCl_2$.....	20	500	Host killed in Dil. of 1/15,000
Wood,[1] $CuSO_4$.....	5	100	Killed host
Quinine sulphate...	2	100	Killed host
Cinchona bark.....	2	100	Killed host
Gentian violet.....	15	100	Dil. 1/100,000, host and protozoa unaffected
Acid fuchsin.....	10	100	Dil. 1/100,000, host and protozoa unaffected
Anhydrous $CaCl_2$[2]..	6	500	Many protozoa killed, host injured
Conc. H_2SO_4[2].....	5	200	Many protozoa killed, host injured
Hornet's nest.....	5	2,000	Host and protozoa unaffected in 2 mos.
Humus.....	10	10,000	Host and protozoa unaffected in 3 mos.
Soil.....	5	4,000	Host and protozoa unaffected in 2 mos.
Soap.....	3	100	Host killed
Leather.....	3	50	Host and protozoa unaffected in 3 weeks
Low temperature...	5	200	2–3° C., no effect in 3 weeks
Starvation.....	20	1,000	Many protozoa killed, host injured
Ether fumes.....	4	100	1 min. no effect on protozoa or host
Fumes of C_2H_5OH.	4	200	2 min. no effect on protozoa or host
Fumes of $CHCl_3$ ...	2	50	1/2 min. no effect on protozoa or host
U.V. Light, 10 in. ..	10	500	15 min. no effect on protozoa or host
X-ray, 2 skin dos...	10	500	No effect on protozoa or host
Inc[4] 35°.5 C. 24 hrs.	15	5,000	Few protozoa killed, host uninjured
Inc[4] 36° C. 24 hrs...	[3]	100,000	All protozoa killed, host uninjured
Inc[4] 42° C. 10 min.	10	500	Few protozoa killed, host uninjured
Inc[4] 43° C. 10 min.	10	500	All protozoa killed, host uninjured
Inc[4] 44° C. 10 min.	10	500	Host not killed
Inc[4] 45° C. 10 min.	10	500	Host not killed
Inc[4] 46° C. 10 min.	10	500	Host not killed
Inc[4] 47° C. 10 min.	10	500	Host nearly all killed
Inc[4] 48° C. 10 min.	10	500	Host all killed, 48° C. is T. D. P. of host

[1] Wood moistened with 5 per cent. Aq. solution of NaCl, KCl, $CaCl_2$ and with many dilutions of $CuSO_4$ and $HgCl_2$

[2] These chemicals were used in dehydration experiments.

[3] More than 2,000 experiments were carried out.

[4] Incubated.

Kalotermes schwarzi, *K. jouteli* and *Prorhinotermes simplex* when incubated for 24 hours at 36° C. are defaunated, but enough material was not available to carry out extensive experiments on these forms as was done with *Reticulitermes flavipes*.

TABLE II.

SHOWING THE LENGTH OF LIFE OF INCUBATED AND NON-INCUBATED TERMITES WHEN FED THEIR NORMAL DIET OF WOOD. 30 EXPERIMENTS SELECTED AT RANDOM FROM A SERIES OF 500.

No. of Experiment.	Treatment.	Approximate No. of Termites Used in Each Experiment.	Length of Life in Days.	Remarks.
1a7	Incubated	4,000	14	
2a7	Unincubated	2,000	60	Jar became too dry due to neglect
11a15	Incubated	500	17	
7a15	Unincubated	1,200	In.[1]	Alive now, 9 months later
12a15	Incubated	400	15	
8a15	Unincubated	1,000	In.[1]	Alive now, 9 months later
13a17	Incubated	300	20	
14a17	Incubated	500	20	
15a17	Incubated	400	21	
1a17	Unincubated	700	In.[1]	Used in other expts. after 6 months
2a17	Unincubated	2,000	In.[1]	Alive now, 8 months later
2a27	Incubated	300	18	
2b27	Incubated	200	21	
3b27	Unincubated	700	In.[1]	Alive now, 5 months later
3a27	Unincubated	500	In.[1]	Alive now, 5 months later
1d27	Incubated	400	7	Mould caused early death
2c27	Incubated	600	15	
1a28	Incubated	200	10	
2a28	Incubated	100	14	
3a28	Unincubated	200	45	Used in other experiments
1a31	Unincubated	1,500	In.[1]	Alive now, 4 months later
2b31	Incubated	500	12	
3b31	Incubated	700	18	
2a31	Incubated	1,000	8	Wood decayed very little
4a31	Incubated	2,000	10	Wood decayed very little
1c32	Unincubated	2,000	In.[1]	Used in other expts. after 3 months
2c32	Incubated	1,500	27	Wood very much decayed
1d32	Unincubated	3,000	In.[1]	Used in other expts. after 3 months
2d32	Incubated	2,000	20	
1f33	Unincubated	1,000	In.[1]	Alive now, 4 months later

To be certain there was no error in the experiments which had been done up to this time, more than five hundred jars of termites were collected; some were incubated and some were not incubated, as shown in Table II. No doubt 100,000 termites

[1] Indefinitely.

were used in these experiments and, in every case, death occurred, as in the preliminary experiments, within 10–20 days on the average after incubation. In no case did the unincubated and faunated controls die. In fact, they are still alive now, more than six months since many of the experiments were completed.

How may the cause of the incubated and defaunated (with the protozoan fauna removed) termites' death be determined? Is death a direct reslt of the incubation? That is, if the termites did not harbor intestinal protozoa which are killed off by the incubation, would death result in 10–20 days? In other words, is the death of the protozoa harbored by the termite in any way connected with or responsible for the death of their host; or is it an entirely independent phenomenon having nothing at all to do with the termite's death. In an effort to answer these questions many experiments were carried out, but only those experiments whose results throw some light on the questions involved will be mentioned and discussed.

Insects, like the cockroach, rather closely related to the termites morphologically, and many wood-boring beetles, rather closely related to termites in habits, were incubated at the same temperature for the same length of time, and for a longer time, as were the termites. These were carefully observed for two months after incubation and no abnormalities were ever observed in any of them. It seems, then, that this temperature is not detrimental to these insects. But the fact that a cockroach or a wood-boring beetle is not killed by the incubation method employed in killing the protozoa of termites, does not show that the termites themselves were not killed directly by incubation, because these insects do not harbor a vast multitude of intestinal protozoa that are suddenly killed off, as do the termites; and besides, they are, after all, quite different in some respects from termites.

Nasutitermes morio Latreille, from Porto Rico, one of the Termitidæ, and, of course, a termite which does not harbor intestinal protozoa, was procured for experimental purposes, the idea being that if this termite could withstand the same incubation temperature that *Reticulitermes flavipes* had been subjected to, and receive no ill effects therefrom, as evidenced by its ability to live indefinitely after incubation, the probability that *Reticulitermes*

flavipes was not killed directly by incubation was very much augmented. Then, some cause other than the effect of the increased temperature on the cells of the termite might be expected to be responsible for the termite's death. But *Nasutitermes morio* could not be kept in closed jars for a longer period than 5–10 days before death occured, regardless of whether they had been incubated or not. *Amitermes* sp. from Uvalde, Texas, another genus of the Termitidæ, was obtained, but it also could not be kept in closed jars. Other genera of the Termitidæ, termites not harboring protozoa in nature so far as known, which might possibly be kept in closed jars—though nothing at all is known regarding this—were not obtainable. It then became necessary to answer in another way the question, does the heating kill the termite directly?

An effort was made to keep the incubated and defaunated termites alive by feeding them substances other than wood, their normal diet. Dextrose, peptone and starch, separately and altogether, were fed them; and it was evident, from the results (see Table III.) obtained by the feeding of these substances, particularly dextrose, that the lives of the incubated and defaunated termites had been prolonged.

But before these experiments had progressed very far a much better method of prolonging the lives of the defaunated termites was discovered, and no more experiments employing dextrose, starch and peptone were carried out. They were placed in glass jars and were fed a diet of fresh humus, which seemed to be a very satisfactory food, as the results tabulated in Table III. show. When they were given humus before any of the characteristic symptoms of wood-fed defaunated termites had appeared, no such symptoms ever appeared. If these symptoms had appeared before humus was fed, the termites after being fed humus for three or four days became much more active, and within six to seven days they were normal again. Even when all the individuals in a jar of defaunated termites were almost dead, due to being fed on wood, many of the almost lifeless ones could be brought back to normal by feeding them humus. The humus fed defaunated termites appeared to be perfectly normal in every way long after all of their wood fed defaunated controls were dead. One hundred jars of defaunated termites were used in the

TABLE III.

SHOWING THE RESULTS OF ATTEMPTS TO PROLONG THE LIVES OF INCUBATED AND DEFAUNATED TERMITES BY FEEDING THEM VARIOUS SUBSTANCES. 27 EXPERIMENTS SELECTED AT RANDOM FROM A SERIES OF 100.

No. of Experiment.	Substance.	Approximate No. of Termites Used in Each Experiment.	Length of Life in Days.	Remarks.
2a15	Dextrose	200	30	
3a15	Dextrose	200	34	
4a15	Dextrose	200	40	
5a15	Peptone	200	4	
10a15	Dextrine	100	30	
7a15	Dex. & Pep.[1]	100	45	
9a15	Dex. & Pep.[1]	100	43	
3a19	Dextrose	150	30	
4a19	Dextrose	200	34	
4f33	Humus	1,000	125	Experiment discontinued[2]
4g33	Humus	1,000	45	Allowed to become too dry
2g33	Humus	500	125	Experiment discontinued[3]
12a35	Humus	1,000	60	Experiment discontinued[3]
13a35	Humus	1,000	60	Experiment discontinued[3]
22a35	Humus	1,000	60	Experiment discontinued[3]
23a35	Humus	1,000	60	Experiment discontinued[3]
36a35	Humus	1,000	40	Changed to a wood diet[4]
20a35	Humus	1,000	45	Changed to a wood diet[4]
19a35	Humus	1,000	20	Changed to a wood diet[4]
9a63	Soil	1,000	50	Experiment not done with care
10a61	Soil	200	45	Experiment not done with care
17a35a	Int. ct.[2]	50	40	Experiment discontinued[3]
17a35b	Int. ct.[2]	50	45	Experiment discontinued[3]
17a35c	Int. ct.[2]	50	34	Experiment discontinued[3]
1a45	F. D. C.[5]	50	60	Experiment discontinued[3]
2a45	F. D. C.[5]	50	60	Experiment discontinued[3]
4a45	F. D. C.[5]	50	60	Experiment discontinued[3]

humus feeding experiments, and from ten to more than a thousand termites were placed in each jar. It does not seem feasible or necessary to state when each of the one hundred jars were collected and incubated and just what happened to each of them, since the results of each experiment are similar, as Table III. shows.

[1] Dextrose and peptone.

[2] Intestinal contents of wood-boring insect larvæ.

[3] No evident signs of death at this time.

[4] Death resulted in 10–20 days later.

[5] Fungus digested cellulose.

(To be continued)